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Reduced Antigenicity of the Hepatitis B Virus HBsAg Protein Arising as a Consequence of Sequence Changes in the Overlapping Polymerase Gene That Are Selected by Lamivudine Therapy

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The prevalence of hepatitis B virus vaccine escape mutants has increased as a consequence of the introduction of global vaccination programs. Furthermore and as a consequence of the organization of the genome of hepatitis B virus (HBV) into overlapping reading frames, the selection of polymerase mutants during long-term lamivudine therapy can select viruses with changes in the overlapping S gene coding for the hepatitis B small antigen (HBsAg). We have investigated the role of lamivudine in selecting HBV mutants with antigenically altered HBsAg protein using pooled human vaccinee sera in enzyme immunosorbent assays and radioimmunoassays. HBsAg proteins containing the vaccine escape mutations G145R and D144E/G145R demonstrated markedly reduced binding to anti-HBs antibody. HBsAg mutants including E164D, W196S, I195M, M198I, and E164D/I195M (corresponding to the polymerase protein changes of V519L, M550I, L526M/M550V V553I, and V519L/L526M/M550V) selected during lamivudine treatment also demonstrated reduced binding to anti-HBs antibody. These findings raise the possibility of lamivudine-resistant mutants arising that possess antigenically distinct HBsAg proteins.

Key Words: hepatitis; mutants; vaccination; lamivudine; polymerase.

INTRODUCTION

Hepatitis B vaccination is an effective means of preventing infection by hepatitis B virus (HBV) in susceptible individuals. The vaccine consists of yeast-derived recombinant hepatitis B small antigen (HBsAg) protein and produces seroconversion in up to 95% of recipients. The HBsAg protein contains the cysteine-rich "a" determinant (Emini *et al.,* 1986; Hauser *et al.,* 1987; Milich, 1997; Peterson *et al.,* 1984; Wallace and Carman, 1997) and it is this region that is recognised by antibodies that are elicited by active hepatitis B vaccination and by anti-HBs antibody present in hepatitis B immune globulin (HBIg) (Jilg *et al.,* 1984; Szmuness *et al.,* 1980).

The antigenicity of the a determinant has been reported to be dependent on the tertiary structure of this region of the protein (Carman *et al.,* 1990; Wallace and Carman, 1997) and other important antigenic epitopes outside of the a determinant and situated on the surface of the virus have also been described (Chen *et al.,* 1996). Some of these epitopes are situated downstream of the a determinant and may also be potential neutralisation domains (Fig. 1) (Chen *et al.,* 1996).

Mutations in and around the a determinant may result in an alteration of the antigenicity of the HBsAg protein and the subsequent failure of anti-HBs antibodies elicited by vaccination to neutralise such mutants (Carman, 1997; Carman *et al.,* 1995, 1996; Coursaget *et al.,* 1987; Hsu *et al.,* 1999; Karthigesu *et al.,* 1994; Swenson *et al.,* 1983; Wallace and Carman, 1997; Wands *et al.,* 1986; Waters *et al.,* 1992). These vaccine escape mutants can infect vaccinated individuals and are prevalent in countries with high endemic rates of HBV infection and with established HBV vaccination programs (Hsu *et al.,* 1999); in Taiwan, for example, up to 28% of children carrying HBV harbour these HBsAg mutants (Hsu *et al.,* 1999). It has been estimated that vaccine escape mutants will become the dominant HBV quasispecies globally (Wilson *et al.,* 2000).

The genome of HBV is organised into overlapping reading frames with the polymerase gene overlapping the envelope (s) gene. As a result of this gene arrangement, HBV isolates with polymerase gene mutations that are selected during the course of antiviral nucleoside analogue therapy may carry altered neutralisation epitopes within the HBsAg. Therapy with lamivudine re-

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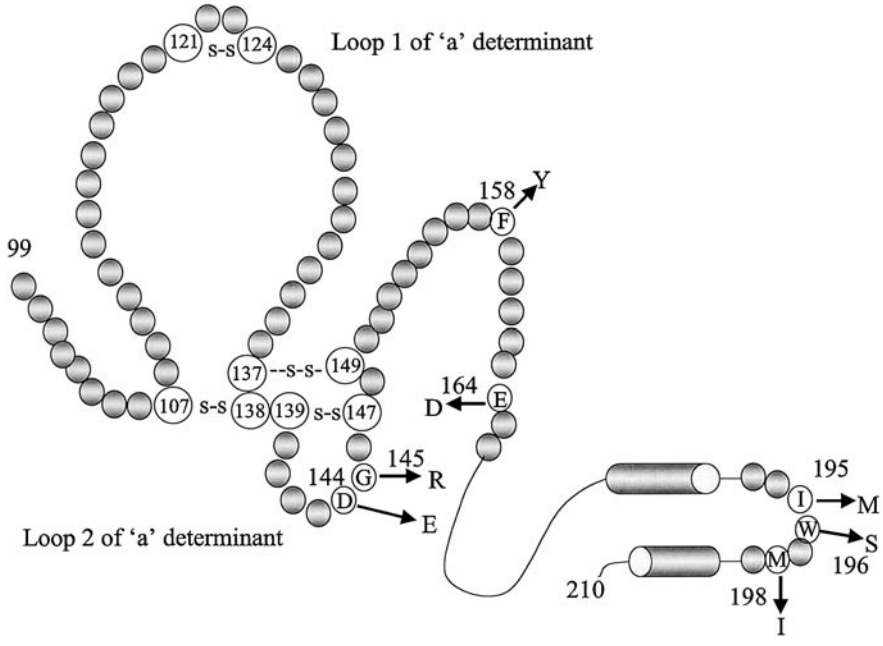

FIG. 1. A diagrammatic representation of the HBsAg protein. The conformation of the two major loops of the "a" determinant is dependent on the presence of a number of important disulphide bridges (-s-s-). The changes of the vaccine escape mutations D144E and G145R are shown in the second loop of the a determinant. The changes selected by lamivudine (F158Y, E164D, I195M, W196S, M198I) occur downstream of the a determinant and are indicated. The carboxy-terminal end of the protein contains two alpha helices (shown here as cylindrical structures), the first of which is present on the surface of the HBsAg particle (Chen *et al.,* 1996). These alpha helices are separated by a turn and held in this conformation by a disulphide bridge. The lamivudine-selected amino acid changes I195M, W196S, and M198I occur within this turn and could disrupt the integrity of this region of the protein. (Adapted from Carman and Wallace, 1997.)

sults in mutations in the polymerase gene, some of which are associated with alterations in the a determinant of the HBsAg protein (Lok *et al.,* 2000); these drug-resistant isolates may have the potential to become vaccine escape mutants. However, an alteration in the antigenicity of these lamivudine-selected HBsAg mutants has not been previously demonstrated.

In this study we used human vaccinee antisera to investigate the differences in antigenicity of the HBsAg protein isolated from HBV mutants selected by lamivudine.

RESULTS

The binding of antibodies present in human vaccinee sera to the mutant HBsAg proteins was assessed using enzyme immunosorbent assays and a radioimmunoassay. Sera were tested individually to define individual variations in antigen–antibody binding and also as pooled samples. The lamivudine-selected mutant proteins will be referred to according to the amino acid changes in the HBsAg. To determine that the quality of antigens produced was consistent, recombinant proteins were analysed by immune electron microscopy and SDS–PAGE. The assembly of wild-type HBsAg protein into 22-nm particles was confirmed by immune electron microscopy (Fig. 2A). A 24-kD protein could be identified for the wild-type HBsAg and each mutant protein by SDS–PAGE and silver staining (Fig. 2B).

Binding of vaccinee serum to recombinant antigens using enzyme immunoassays (EIA)

The binding of antibody present in individual vaccinee sera to recombinant HBsAg in an EIA demonstrated that the binding to mutant proteins was usually reduced rel-

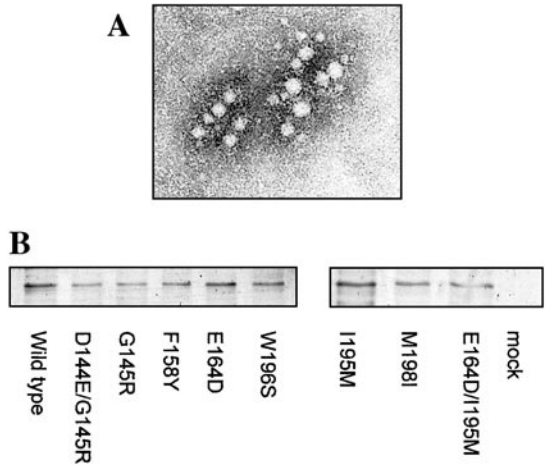

FIG. 2. (A) Analysis of the quality of the HBsAg proteins produced. After the production and purification of recombinant antigens, immune electron microscopy of the wild-type HBsAg protein was performed with goat anti-HBs antibody to demonstrate that proteins had assembled into 22-nm particles. (B) Wild-type and mutant recombinant HBsAg proteins were also separated by electrophoresis in 12% SDS polyacrylamide gels and protein bands detected by silver staining of gels.

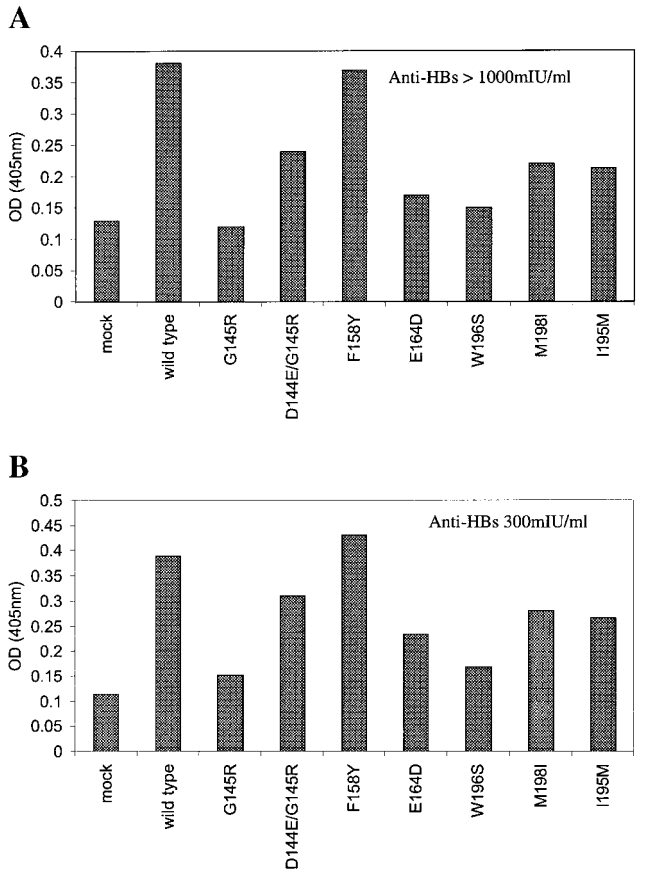

FIG. 3. Binding of individual vaccinee sera to wild-type and recombinant yeast-derived HBsAg. EIA plates were coated with 5 μg/ml of recombinant HBsAg and incubated with individual human vaccinee sera. Anti-HBs antibody titres in serum samples were determined by IMX (Abbott). The binding pattern of two representative sera to the mutant proteins is shown. Anti-HBs antibody was present at saturating levels and despite this failed to bind to several mutant HBsAg proteins.

ative to the binding to the wild-type HBsAg (Fig. 3). This was evident with the binding to the mutants carrying the G145R and D144E/G145R amino acid changes and that are recognised as vaccine escape mutants. The vaccinee sera also showed varying degrees of reduced binding to the HBsAg mutants selected during lamivudine treatment. This was most apparent with the W196S and the E164D mutants and to a lesser degree with the M198I and I195M mutants. The binding of vaccinee sera to the F158Y mutant was similar to the wild-type protein. Some slight individual variation in the peak levels of antibody binding to the different HBsAg proteins was noted with different serum samples tested (compare Figs. 3A and 3B) but this did not correlate with the serum anti-HBs antibody titre that was measured against wild-type HBsAg (>1000 IU/ml versus 300 IU/ml) (Fig. 3). Of the nine individual vaccinee sera examined, the pattern of binding of anti-HBs antibody was similar (Fig. 3 and data not shown), although some sera did appear to bind more strongly to the mutant HBsAg proteins, including the dual D144E/G145R and the lamivudine-selected mutants E164D and I195M. These results indicate that antisera with high-serum anti-HBs antibody titres are unable to recognise these mutants effectively.

Titration of wild-type and mutant antigen binding by MEIA

Binding of the recombinant HBsAg proteins was also tested using a commercial microparticle EIA (IMX HBsAg, Abbott Laboratories, North Chicago, IL). Serial twofold dilutions of antigens were performed and samples analysed by IMX assay according to the manufacturer's instructions. The mutant HBsAg proteins were poorly bound using this assay. A marked reduction in binding with the vaccine escape mutant G145R was detected (Fig. 4). In comparison to the wild-type HBsAg, the lamivudine-selected mutants bound poorly antibody in this assay (Fig. 4). The binding of anti-HBs antibody binding to the lamivudine mutants was variable but was reduced with all of the mutant HBsAg proteins tested (Fig. 4). These findings supported those of the EIA using human vaccinee sera and demonstrated that the amino acid

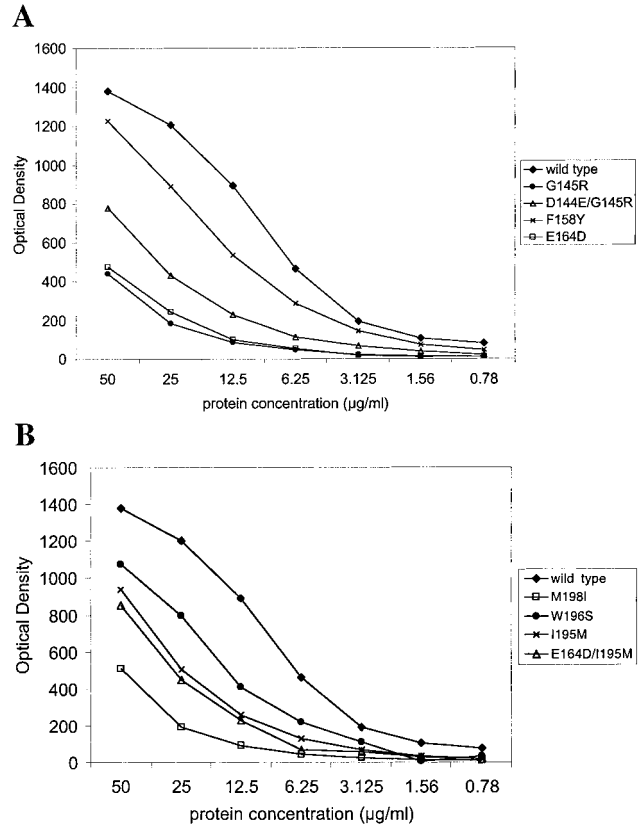

FIG. 4. Binding of recombinant HBsAg proteins to anti-HBs antibodies in a commercial microparticle EIA. Twofold serial dilutions of the proteins were performed and the ability to detect the wild-type and mutant proteins assessed in the Abbott IMX assay. The binding of the wild-type and the vaccine escape G145R HBsAg proteins to antibodies in the IMX MEIA are shown (A). The lamivudine HBsAg mutants had a reduced level of binding than the wild-type HBsAg (A and B).

changes in HBsAg selected as a consequence of lamivudine treatment are sufficient to change the antigenicity of the HBsAg.

Binding of mutant HBsAg proteins to vaccinee anti-HBs antibodies by Radioimmunoassay (RIA)

To determine the antigenic relatedness of the HBsAg proteins, the ability of the mutant HBsAg proteins to inhibit the binding of anti-HBs antibody to wild-type HBsAg protein was determined by competitive antigen–antibody binding studies. The binding of anti-HBs antibodies present in vaccinee sera to wild-type HBsAg in the solid phase by RIA was markedly inhibited by the homologous recombinant wild-type HBsAg protein and also by the F158Y mutant (Fig. 5). In contrast, the lamivudine-selected mutants E164D, I195M, M198I, and E164D/I195M poorly inhibited the binding of anti-HBs antibody to the wild-type HBsAg and as a consequence a higher level of unbound anti-HBs antibody was detected in the RIA (Fig. 5). The IC_{50} for the inhibition of binding of anti-HBs antibody to wild-type HBsAg in the solid phase of the RIA is summarised in Table 1. Little or no inhibition was observed with the vaccine escape mutant proteins G145R and D144E/G145R. The greatest increase in the IC_{50} was observed with the vaccine escape mutants G145R and D144E/G145R and the E164D/I195M mutant. A moderate increase was observed with all polymerase mutants except for F158Y (Table 1).

TABLE 1

Binding of Anti-HBs Antibody in Pooled Vaccinee Sera to Recombinant HBsAg Proteins Using the AUSAB RIA

HBV mutants	IC_{50} (μg/ml)
Wild type	1.09[a]
G145R	>55.0
D144E/G145R	>55.0
F158Y	1.86
E164D	14.86
W196S	8.29
M198I	12.5
I195M	5.26
E164D/I195M	54.53

[a] Amount of protein required to effect a 50% reduction in the binding between wild-type HBsAg and anti-HBs antibody in pooled vaccinee serum.

DISCUSSION

Reduced binding of mutant HBsAg proteins to anti-HBs antibody

The commercially available HBV vaccines which contain recombinant yeast-derived HBsAg demonstrate efficacy in preventing hepatitis B infection (Emini *et al.,* 1986; Hadler *et al.,* 1986; Hauser *et al.,* 1987; Hitzeman *et al.,* 1983; Stevens *et al.,* 1987; Valenzuela *et al.,* 1982). The levels and durability of the anti-HBs antibody response elicited by the recombinant yeast vaccine, however, are lower than those achieved with the HBsAg vaccine derived from the plasma of infected individuals (Jilg *et al.,* 1988; Papaevangelou *et al.,* 1985; Stevens *et al.,* 1987). Hepatitis B virus mutants carrying amino acid sequence changes within the a determinant are not neutralised by antibodies induced by the recombinant vaccine and cause infection in vaccinated individuals (Carman *et al.,* 1995, 1990; Coursaget *et al.,* 1987; Hsu *et al.,* 1999; Karthigesu *et al.,* 1994; Linnemann and Askey, 1984; Oon *et al.,* 1995; Swenson *et al.,* 1983; Wands *et al.,* 1986).

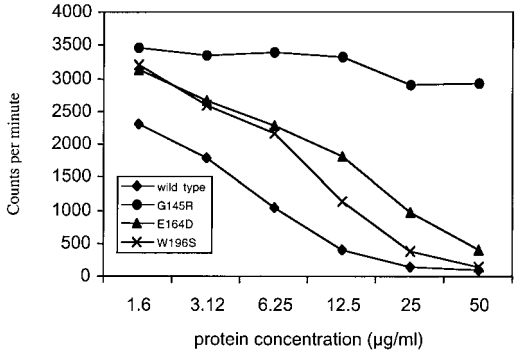

FIG. 5. Binding of anti-HBs antibodies to wild-type HBsAg-coated beads in the presence of mutant HBsAg. Serial dilutions of mutant HBsAg antigens were incubated with a limiting dilution of anti-HBs antibody from pooled vaccinee sera. Supernatants were then incubated with HBsAg-coated beads. The ability of the recombinant mutant HBsAg proteins to bind to anti-HBs antibody was analysed by determining the amount of residual unabsorbed anti-HBs antibody using the AUSAB RIA (Abbott). The binding of the E164D and W196S lamivudine mutants is shown in comparison to the wild-type and the G145R vaccine escape mutant.

In this study we have shown that changes within the HBsAg protein downstream of the a determinant arising from lamivudine-selected mutations result in antigenically distinguishable species. The level and pattern of binding of individual vaccinee sera to mutant HBsAg proteins was similar. In addition, the reduction in the antibody binding to the mutant HBsAg proteins was evident despite the presence of saturating levels of anti-HBs antibody. This suggests that the amino acid changes in the HBsAg selected by lamivudine alter the antigenicity of the HBsAg and therefore the ability of anti-HBs antibody to recognise and bind to the protein.

Lamivudine-selected mutations in the S gene have been identified by sequencing HBV isolates from the serum of patients on long-term lamivudine (Lok *et al.,* 2000; Yeh *et al.,* 2000). To date it has only been assumed that these mutations may have the potential to alter the antigenicity of the HBsAg protein. Our findings demonstrate that lamivudine-selected mutations in the HBsAg produce a decrease in the antigenicity of the protein. Whether these changes produce HBV species that are not neutralised by vaccine induced anti-HBs antibody and therefore result in infection in vaccinated individuals is

yet to be established. However, our findings raise the possibility that lamivudine-selected HBsAg protein changes may have the potential to escape neutralisation by anti-HBs antibody.

We used a variety of commonly performed assays to demonstrate these antigenic differences. In the assays used, the G145R and D144E/G145R vaccine escape mutant HBsAg proteins demonstrated reduced binding to vaccinee anti-HBs antibody. In EIAs, the binding of the individual vaccinee sera to the HBsAg mutant proteins was most reduced with the E164D, I195M, W196S, and M198I lamivudine-selected mutants. The binding of the mutant F158Y was not reduced.

The different pattern of binding was confirmed when antigens were tested with a commercial microparticle EIA. With this assay, the binding of the polymerase-selected HBsAg mutants to anti-HBs antibody was reduced in comparison to the wild-type HBsAg protein (Fig. 3). Antibody binding to the G145R mutant was markedly reduced.

The binding of pooled vaccinee anti-HBs antibodies to antigens in their native state in solution using a commercial RIA also revealed a comparable pattern in antibody binding. These assays were used as competitive radioimmunoassays to determine if the binding of pooled vaccinee anti-HBs antibodies to wild-type HBsAg could be inhibited by preincubating the sera with the mutant HBsAg proteins. Both vaccine escape mutants, G145R and D144E/G145R, and the lamivudine-selected mutant E164D/I195M demonstrated minimal inhibition of binding of anti-HBs antibody to wild-type HBsAg. Similarly, the E164D, M198I, I195M, and W196S lamivudine-selected mutants also showed reduced binding to the anti-HBs antibodies and therefore poorly inhibited their interaction with wild-type HBsAg.

The binding of anti-HBs to HBsAg in the *in vitro* assays used may be dependent not only on the protein and epitope sequence but also on the manner in which the epitopes are presented in the assay. In all of the assays used in this study, the G145R vaccine escape mutant HBsAg protein constantly demonstrated reduced binding to vaccinee anti-HBs. Nevertheless, differences in the patterns of binding were apparent using solid-phase ELISA assays where wild-type and mutant HBsAg proteins were coated onto a solid phase under alkaline conditions compared with results obtained with antigen–antibody binding studies performed in solution. It is possible that exposure of additional epitopes or sequestration of epitopes by a process of partial denaturation of antigens produced a different pattern of binding than proteins in solution. Conformational preferences of epitopes in fluid or solid phase has been previously described and may play an important role in epitope functionality (Aleanzi *et al.,* 1996).

Using a commercial microparticle sandwich EIA, the reduction in binding of the HBsAg mutants to anti-HBs was greatest with the G145R, M198I, E164D, I195M, and E164D/I195M mutants, whereas using a commercial RIA based on competitive binding, the binding of anti-HBs was markedly reduced with the E164D and W196S mutants and the vaccine escape mutant, G145R, demonstrated minimal binding to vaccinee anti-HBs.

It should be emphasized however, that despite the differences observed in the pattern of binding of antibody to the mutant HBsAg proteins using the different assays, the binding of the lamivudine-selected HBsAg protein mutants was consistently reduced when compared to wild-type protein. Furthermore, these differences were not the consequence of variability in the quality of mutant antigens produced. All antigens were produced under identical conditions. The presence of 24-kD protein was confirmed by SDS–PAGE and silver staining and assembly into 22-nm particles confirmed by immune electron microscopy (Fig. 2).

Vaccine escape HBV mutants

Vaccine escape HBV mutants have been reported previously (Carman *et al.,* 1995; Karthigesu *et al.,* 1994; Wallace and Carman, 1997) and common vaccine escape variants include G145R, D144A, P142S, Q129H, I/T126N/A, and M133L (Wallace and Carman, 1997). Furthermore, both monoclonal and polyclonal hepatitis B immune globulin can select escape mutants, including A105P, S/T114R, K115T, T118A, Y/C124R, P120E/T, T122N, Q129H, G130R, N131S, C138Y, S140T, K141I, P142S, D144A, G145R, and C149R (Wallace and Carman, 1997). All of these mutants carry changes within the a determinant and cause hepatitis in fully vaccinated individuals (Carman, 1997; Carman *et al.,* 1996, 1995; Coursaget *et al.,* 1987; Karthigesu *et al.,* 1994; Swenson *et al.,* 1983; Wallace and Carman, 1997; Wands *et al.,* 1986; Waters *et al.,* 1992). The G145R variant is associated with high levels of viraemia and persistence for periods as long as 8 years, suggesting that this is a stable mutant.

Lamivudine-selected HBsAg protein changes

The HBsAg protein changes that are selected by lamivudine therapy occur in regions of the HBsAg protein that are situated on the surface of the virus and are potentially recognised by anti-HBs antibodies (Fig. 1) (Chen *et al.,* 1996). Some of these mutations, for example, those corresponding to the I195M, W196S, and the M198I mutants, affect a conformational region of the HBsAg protein consisting of two short alpha helices separated by a disulphide bridge forming a turn (Fig. 1) (Chen *et al.,* 1996). Sequence changes within this region may alter the structural integrity of the protein and alter binding to anti-HBs antibody without affecting the assembly or secretion of the virus (Prange *et al.,* 1995). In addition an alteration of one epitope of the a determinant may alter

TABLE 2

Mutants of HBV and a Comparison of Their Amino Acid Changes within the Wild-Type Polymerase and HBsAg Proteins[a]

	Position within the polymerase protein at which sequence change occurs						Position within the HBsAg protein at which sequence change occurs						
Mutant virus	499	512	519	526	550	553	144	145	158	164	195	196	198
Wild type	G/W	F	V	L	M	V	D	G	F	E	I	W	M
D144E/G145R	E						E	R					
G145R	Q							R					
F158Y		L							Y				
E164D			L							D			
I195M					V						M		
I195M				M	V						M		
W196S					I							S	
M198I						I							I
E164D/I195M		L	M	V						D			

[a] Positions within the wild-type HBV polymerase and HBsAg at which sequence changes occur within the mutant proteins. The amino acid changes are shown in single letter code for the individual mutants.

the binding of antibody to proximal but distinct epitopes (Waters *et al.,* 1992).

The nature of the anti-HBs antibody response elicited after vaccination may also vary considerably depending on the source of HBsAg (Heijtink *et al.,* 2000). The binding characteristics of anti-HBs antibody elicited by vaccination with the yeast-derived vaccine are different from those induced by plasma-derived HBsAg immunisation (Heijtink *et al.,* 2000), suggesting that the presentation of immune-reactive epitopes of the yeast-derived HBsAg is quite different than those on HBsAg in an infected individual (Heijtink *et al.,* 2000). The efficacy of the yeast-derived vaccine may therefore be reduced in the event of a challenge with hepatitis B virus, especially if that virus carries an antigenically altered HBsAg protein.

The clinical significance of the lamivudine-selected changes within the S gene is not known but mutations within the HBsAg protein that arise during the course of lamivudine therapy have been reported (Lok *et al.,* 2000). The widespread use of nucleoside analogues such as lamivudine may have the potential to select HBV mutants that are poorly bound by vaccine-induced anti-HBs antibody. Further investigations are warranted to determine if these HBsAg protein changes will result in infection of vaccinated individuals and potentially reduce the efficacy of the current HBV vaccine.

MATERIALS AND METHODS

Mutagenesis of the S gene

HBsAg mutants were produced by site-directed mutagenesis using an infectious clone of HBV that includes all of the major reading frames of HBV. Mutagenesis was performed using the Quickchange Site Directed Mutagenesis system, according to the manufacturer's instructions (Stratagene, La Jolla, CA). The HBsAg mutations were obtained from a HBV sequence database developed at the Victorian Infectious Diseases Reference Laboratory (VIDRL) based on viral isolates from the sera of patients treated with lamivudine. Several of these mutants have been previously reported (Bartholomew *et al.,* 1997; De Man *et al.,* 1998; Ling and Harrison, 1999; Tipples *et al.,* 1996; Wallace and Carman, 1997). The S gene mutations with the respective changes in the overlapping polymerase gene were confirmed by DNA sequencing. The sequence changes of HBV mutants are summarised in Table 2.

Plasmid construction

The full-length S genes carrying potential vaccine escape mutations were amplified by PCR using primers with 5′ *Sma*1 and 3′ *Cla*1 restriction sites. The PCR fragments were separated by agarose gel electrophoresis and purified using a Prep-A-Gene kit (Bio-Rad, CA) according to the manufacturer's instructions. The 5′ primer was designed to introduce a hexahistidine tag at the amino terminal end of the S protein. The primer sequences used are as follows: Forward primer: 5′TCC CCC GGG CAT CAT CAT CAC CAT CAC ATG GAG AAC ATC ACA TCA GG 3′; Reverse primer: 5′CCA TCG ATT TAG GGA ATA ACC CCA TC 3′. The reverse primer used for the M550I mutant was identical to the primer sequence above except that it included a 3′ *Kpn*1 restriction site. The sequence of this primer (reverse primer 2) was 5′CGG GAT CCT TAG GGA ATA ACC CCA TC 3′. Purified S gene products were subcloned into pCRScript (Stratagene) with 100 u of T4 DNA ligase (New England Biolabs, MA) supplemented with 1 mM ATP. Plasmids were transformed into *Escherichia coli* (DH5α) by heat

shock and fidelity of the clones confirmed by DNA sequencing.

The hexahistidine-tagged S genes were digested with *Sma*1 and *Cla*1 (or *Kpn*1 for the M550I mutant), separated by agarose gel electrophoresis, purified with Prep-A-Gene (Bio-Rad), and finally subcloned into the yeast expression vector yEPpFlag (Eastman Kodak Co.). Fidelity of the clones was again confirmed by DNA sequencing.

Transformation of yeast cells

Wild-type and mutant S genes were transformed into *Saccharomyces cerevisia* using PEG–bicine transformation under tryptophan selection according to the manufacturer's instructions (Eastman Kodak Co.). Briefly, BJ3505 cells (*S. cerevisiae*) were grown for 24 h at 28°C in 10 ml YPD medium (2% glucose, 2% peptone, 1% yeast extract). This culture was used to inoculate a further 90 ml YPD medium and incubated at 28°C for 4 h or until the OD_{600} had reached a value of 0.6. An aliquot of cells (10 ml) was centrifuged for 5 min at 3200 *g* (Beckman GPR centrifuge) and resuspended in 5 ml SBEG medium (1 M sorbitol, 10 mM bicine, pH 8.35, 3% polyethylene glycol 1000, Sigma), and the cells sedimented by centrifugation for 5 min at 3200 *g*. The pellet was resuspended in 200 μl of SBEG medium before incubating at 28°C for 5 min without shaking.

An amount (1.0 μg) of plasmid DNA of each mutant was added to separate aliquots of cells in SBEG before incubating for a further 10 min at 28°C with shaking. The transformation mixture was incubated at −80°C for a further 45 min, followed by thawing at 37°C and incubating for 1 h without shaking. Following this incubation, 2 ml NB buffer (0.15 M NaCl, 10 mM bicine, pH 8.35) was added and the cells sedimented by centrifugation as above. The cells were resuspended in 500 μl NB buffer and a 100-μl aliquot plated on selective CSM-Trp plates [0.67% yeast nitrogen base (without amino acids), 2% dextrose, 0.074% CSM-Trp amino acid mixture (Bio 101), 100 mg/l glutamic acid, 400 mg/l serine, 2% agar].

Expression of recombinant HBsAg proteins

Recombinant HBsAg proteins were expressed by inoculating 10 ml CSM-Trp medium and incubating for 48 h at 28°C with shaking. A 2.5-ml aliquot of this culture was used to inoculate 47.5 ml YPSHM medium (1% dextrose, 3% glycerol, 1% yeast extract, 8% peptone, 20 mM calcium chloride) and incubated for 72 h at 28°C with shaking at 175 rpm.

Recovery of HBsAg by nondenaturing lysis of yeast cells

Following the expression of HBsAg proteins, 50 ml cultures of yeast cells were sedimented by centrifugation for 15 min at 3200 *g*. The pellet was resuspended in 1 ml disruption buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.0 with 0.1% Triton X-100). A 400-μl aliquot of resuspended cells was transferred to a 10-ml tube and 1.6 ml glass beads was added. The mixture was vortexed for 1 min and then incubated on ice for 2 min. These two steps were repeated five times before allowing the glass beads to settle and before collecting the supernatant (cell lysate). A further 1.5 ml disruption buffer was added to the lysate and the mixture inverted 10 times before allowing the glass beads to settle. The supernatant was collected and the cell lysates clarified by centrifugation for 1 h at 10,000 *g*. An aliquot of the supernatant was tested for HBsAg using a commercial microparticle enzyme immunoassay (MEIA; IMX, Abbott Laboratories) before proceeding with purification of recombinant proteins.

Protein purification

The recombinant HBsAg proteins were purified by immobilised metal affinity chromatography using Talon resin (Clontech, Palo Alto, CA). A 4-ml volume of resin was washed in 50 mM NaH_2PO_4 with 300 mM NaCl, pH 7.0 before sedimenting the resin for 5 min at 700 *g*. The clarified cell lysate was added to the resin and gently mixed for 20 min at room temperature. The mixture with the hexahistidine-tagged HBsAg bound to the resin was centrifuged for 5 min at 700 *g*. The resin was then washed with 10 bed volumes of wash buffer (50 mM NaH_2PO_4 with 300 mM NaCl, pH 7.0) at room temperature for 10 min on a rotating wheel before centrifugation for 5 min at 700 *g*. The supernatant was discarded and the resin washed a further two times. The resin mixture was resuspended in one bed volume of wash buffer and transferred to a gravity-flow column. The resin was washed twice with five bed volumes of wash buffer followed by three washes with wash buffer containing 10 mM imidazole and one final wash with wash buffer containing 25 mM imidazole. The hexahistidine-tagged HBsAg protein was finally eluted and collected in 500-μl fractions using four bed volumes of elution buffer (45 mM NaH_2PO_4, 150 mM NaCl, 100 mM imidazole, pH 7.0). An aliquot of each eluate was tested for HBsAg using the commercial IMX assay (Abbott). Proteins in strongly reactive fractions were used in an EIA format or concentrated by ultrafiltration using Centricon 30 filters (Amicon, MA). Analysis of proteins by SDS–PAGE and silver staining demonstrated that proteins were >90% pure. All proteins were quantitated by Lowry assay (Protein DC, Bio-Rad). The quality of proteins produced was analysed by SDS–PAGE and silver staining with Silver Stain Plus (Bio-Rad) according to the manufacturer's instructions. Finally, immune electron microscopy was performed to ensure that the HBsAg proteins had formed 22-nm particles. In brief, recombinant HBsAg was immunoprecipitated overnight at 4°C with goat anti-HBs antibody (Dako,

CA). Immune complexes of anti-HBs–recombinant HBsAg were stained with 3% phosphotungstic acid (pH 7.0) on a 400-mesh Formvar carbon-coated copper grid and visualized using a CM12 STEM electron microscope (Philips, Eindhoven, Holland).

Binding of antibodies to mutant HBsAg proteins in an enzyme immunoassay

Sera were collected from volunteers who had been vaccinated with commercially available HBV vaccines (HBVax II, Merk Sharp Dohme or Engerix B, Glaxo Smith Kline). The binding of anti-HBs antibody to recombinant HBsAg was analysed by EIA. Plates (Maxisorp, Nunc) were coated with recombinant antigens at the concentrations indicated. All antigens were prepared in a coating buffer (15 mM Na_2CO_4, 35 mM $NaHCO_3$, 0.21 mM NaCl, pH 9.4) and held overnight at 4°C.

The antigen solutions were removed and plates incubated for 1 h at 37°C with blocking solution [10% skim milk powder and 0.5% Tween 20 in phosphate-buffered saline (PBS)]. Human vaccinee sera and goat anti-human horseradish peroxidase (HRP) conjugate (Dako) were prepared in antibody diluent buffer (10% skim milk powder and 0.5% Tween 20 in PBS). Vaccinee sera were incubated with antigen-coated plates for 2 h and the anti-human–HRP conjugate (Dako) was incubated for 1 h at room temperature. Plates were washed using PBS containing 0.05% Tween 20 between these treatments. Absorbance was read at 405 nm.

Titration of recombinant HBsAg protein binding by IMX MEIA

The panel of wild-type and mutant recombinant HBsAg proteins was examined for antigenicity using a commercial microparticle enzyme immunoassay (IMX, MEIA, Abbott Laboratories) according to the manufacturer's instructions. Briefly, serial dilutions of the proteins were prepared in PBS and added to monoclonal anti-HBs antibody-coated microparticles. Biotinylated goat anti-HBs antibody was added, forming antigen–antibody complexes bound to microparticles. The complexes are bound to a glass fibre matrix and rabbit anti-biotin–alkaline phosphatase conjugate added, followed by the substrate 4-methylumbelliferyl phosphate. Fluorescence was then measured by the MEIA optical assembly provided by Abbott Laboratories.

Binding of antibody to mutant HBsAg proteins by RIA

Sera were collected from volunteers who had been vaccinated with recombinant HBV vaccine. Sera were pooled and anti-HBs antibody titres determined using the IMX assay. A limiting dilution, determined by performing serial twofold dilutions of serum prepared in anti-HBs antibody negative serum, was defined as the highest dilution of pooled vaccinee sera that gave an antibody titre one dilution above the cutoff of the anti-HBsAg IMX assay. Limiting dilutions of pooled vaccinee sera were then incubated with serial dilutions of wild-type or mutant recombinant HBsAg proteins for 1 h at 37°C. The difference in the binding of anti-HBs antibody to wild-type and mutant HBsAg proteins was then determined by detecting residual anti-HBs antibody, i.e., that which was not absorbed by the recombinant HBsAg proteins, by incubating samples overnight with HBsAg-coated plastic beads from the AUSAB RIA kit (Abbott Laboratories) according to the manufacturer's instructions. Briefly, following an overnight incubation, residual anti-HBs antibody is bound to the HBsAg-coated beads. After exhaustive washing, only antibody bound directly to the HBsAg-coated beads remains and this is detected by the addition of ^{125}I-labelled anti-human IgG and bound ^{125}I-labelled HBsAg determined.

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